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Enterococcus faecium: from evolutionary insights to practical interventions

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Evaluation of the Xpert *vanA/vanB* assay using enriched inoculated broths for the direct detection of *vanB* VRE

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ABSTRACT:

Rapid and accurate detection of VRE (vancomycin resistant enterococci) is required for adequate antimicrobial treatment and infection prevention measures. Previous studies using PCR for the detection of VRE, including the Cepheid's Xpert *vanA*/*vanB* assay, reported accurate detection of *vanA* VRE, however many false positive results were found for *vanB* VRE. This is mainly due to non-enterococcal *vanB* genes which can be found in the gut flora. Our goal was to optimize the rapid and accurate detection of *vanB* VRE and to improve the positive predictive value (PPV) by limiting false-positive results. We evaluated the use of the Xpert *vanA*/*vanB* assay on rectal swabs and on enriched inoculated broths for the detection of *vanB* VRE. By adjusting the cut-off CT-value to ≤ 25 for positivity by PCR on enriched broths, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) resulted in 96.9%, 100%, 100% and 99.5% for *vanB* VRE, respectively. As shown in this study CT-values ≤ 25 acquired from enriched broths can be considered as true-positive. For broths with CT-values between 25-30, we recommend to confirm this by culture. CT-values of >30 appeared to be true-negative. In conclusion, this study shows that the Cepheid's Xpert *vanA*/*vanB* assay performed on enriched inoculated broths with an adjusted cut-off CT-value is an useful and rapid tool for the detection of *vanB* VRE.

INTRODUCTION

Vancomycin resistant enterococci (VRE) have emerged as an important nosocomial problem worldwide. The rise of VRE is mostly due to *Enterococcus faecium*, with *vanA* and *vanB* being the two clinically most important genotypes [1]. VanA-type resistance is induced by teicoplanin and vancomycin causing resistance to both antibiotics. In contrast, VanB-type resistance is only induced by vancomycin, resulting in variable levels of vancomycin resistance but still being susceptible to teicoplanin [2].

Rapid and accurate detection of VRE is required for adequate antimicrobial treatment and infection prevention measures. Culture based methods to detect VRE are often time-consuming and take several days to complete (2-5 days). These time-consuming methods have a high economic impact on the infection control measures that has to be taken by the hospital, especially during outbreaks [3]. Several studies evaluated PCR-based methods for rapid detection of VRE including the Cepheid's Xpert *vanA*/*vanB* assay [4-7]. This assay runs on the Cepheid GeneXpert™ system, a fully automated processor that combines DNA extraction, real-time PCR amplification and detection, providing results within an hour. PCR-based methods are highly sensitive and specific for the detection of *vanA* VRE [7]. However, for *vanB* VRE many false positive results are reported, mainly due to non-enterococcal *vanB* genes which can be found in the gut, especially in anaerobic bacteria like *Clostridium species* [8-11]. Therefore, positive *vanB* VRE results still need to be confirmed by culture. An additional problem is that VanB-type resistance is sometimes difficult to detect since the vancomycin minimum inhibitory concentration (MICs) can be below the antimicrobial susceptibility breakpoint of ≤4 mg/L defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [12-14].

In our hospital, VRE screening is performed in patients coming from foreign hospitals, on ICU wards and in case of an unexpected VRE observation, e.g. if VRE is found in clinical specimens from epidemiologically linked patients. Isolation precautions are applied to patients coming from foreign hospitals at admission until patient samples are negative. During a (suspected) VRE outbreak patients are cohorted and screened on regular bases. Prior to this study, VRE detection was performed on in enriched inoculated broths with a conventional gel-based PCR. However, many false-postive results were obtained with this technique.

In March and April 2013 our hospital faced an outbreak with *vanB* VRE. During this outbreak we used and evaluated the Xpert *vanA*/*vanB* assay on rectal swabs and on enriched inoculated broths. Our goal was to optimize the rapid and accurate detection of *vanB* VRE and to improve the positive predictive value (PPV) by limiting false-positive results.

METHODS:

The University Medical Center Groningen is a 1300-bed tertiary care center. During an outbreak of *vanB* VRE in March and April 2013, rectal Eswabs (Copan ESwab™) were collected from hospitalized patients at the relevant wards for VRE testing.

Lab-study design:

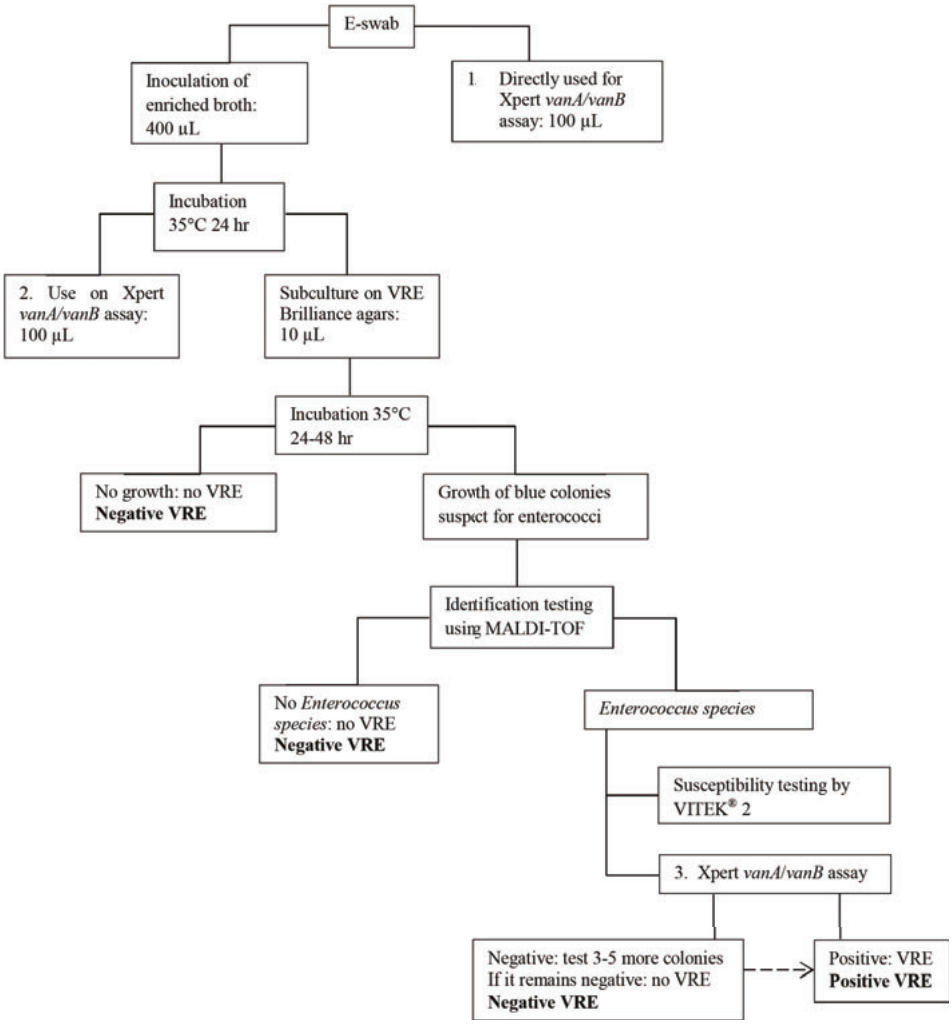
In total 235 Eswabs from 91 patients were used. The Xpert *vanA/vanB* assay was directly performed on 100 µL Eswab medium, and on enriched inoculated broths after 24 hours of incubation. For this latter, 9 mL of Brain Heart Infusion (BHI) broth containing amoxicillin 16mg/L, amphoterin-B 20 mg/L, aztreonam 20 mg/L and colistin 20 mg/L was inoculated with 400 µL Eswab medium and incubated at 35°C for 24 hours. Amoxicillin was used since VRE outbreaks are typically caused by amoxicillin resistant enterococci (ARE) that acquired resistance for vancomycin [15, 16]. Moreover, amoxicillin limits the growth of amoxicillin sensitive anaerobic bacteria like *Clostridium species* which are one of the most relevant species that may contain *vanB* genes [8, 17, 17]. Vancomycin was not added to the broth, as this would hamper the detection of *vanB* VRE expressing low vancomycin MICs [12, 14]. From the broths, 100 µL was used in the Xpert *vanA/vanB* assay and 10 µL of broth was subcultured on VRE Brilliance agars (Oxoid®). Agars were incubated at 35°C and examined after 24-48 hours. Blue colonies suspected for enterococci were identified by MALDI-TOF Mass Spectrometry (Bruker). Confirmed *Enterococcus species* were tested for antibiotic susceptibility using VITEK®2 (Biomerieux). The MIC clinical breakpoints defined by the (EUCAST) for *Enterococcus* spp. are as follows: for vancomycin, susceptible, ≤4 mg/L; resistant, >4 mg/L; for teicoplanin, susceptible, ≤2 mg/L; resistant, >2 mg/L [17]. Subsequently colonies were analyzed in the Xpert *vanA/vanB* assay. In case the Xpert vanA/van B assay was negative, 3-5 more colonies were tested (Figure 1).

The Cepheid GeneXpert™ system:

The Xpert *vanA/vanB* assay was performed on three different sources as depicted in Figure 1. For Eswab medium as well as for enriched inoculated broths, 100 µL was added to the elution buffer, vortexed for 10 seconds and transferred into the Xpert *vanA/vanB* cartridge. For isolates on VRE Brilliance agar, a suspension of 1-2 bacteria colonies was made using 1 mL milli-Q water (Sigma-Aldrich™), vortexed for 10 seconds and then diluted 1:500 in milli-Q water. Subsequently, 75 µL of the diluted sample was added to the elution buffer and transferred to the Xpert *vanA/vanB* cartridge. The amounts used for the Xpert *vanA/vanB* assay were

advised by the manufacturers' and/or validated in our laboratory. Further procedures were performed according to the manufactures' guidelines (Xpert *vanA/vanB* 301-0188). According to these guidelines CT-values of ≤36 are considered to be positive, whereas CT-values of >36 are considered to be negative. A CT-value of 0 for *vanA* and *vanB* indicates no amplification and is considered to be negative if the internal control present in the assay is valid.

Figure 1: Workflow of the study, including definitions of negative and positive VRE indicated in bold (gold standard). Numbers 1, 2 and 3 reflect the three different sources used in the Cepheid's Xpert *vanA/vanB* assay.



Resolution of discordant results:

As mentioned before, VRE detection is difficult and different detection methods are used. The use of a direct rectal swab culture method for the detection of VRE colonization shows a high rate of false-negative results [18]. Culture of rectal samples after broth enrichment followed by species identification and susceptibility testing is most sensitive for detecting VRE [19]. Therefore, we defined a sample as true-positive for VRE when the Xpert assay performed on the isolate from the VRE Brilliance agar, after broth enrichment, was positive (our gold standard). A sample was considered as true-negative for VRE in case of the following results 1) observation of no growth on VRE Brilliance agar after broth enrichment, 2) growth of species other than *Enterococcus species* on the VRE Brilliance agar 3) growth of *Enterococcus species* on VRE Brilliance agar but negative in the Xpert assay performed on the isolate (Figure 1).

The new algorithm

Based on the CT-values acquired from Eswab medium compared to those acquired from the enriched inoculated broths, we defined a new cut-off value for positivity by PCR on enriched broths. Broths with CT-values ≤ 25 were considered to be true-positive for *vanB* VRE, whereas broths with CT-values between 25-30 require confirmation by culture. Samples with CT-values of 0 (no amplification) or >30 were considered to be negative. To test our new algorithm, we prospectively evaluated 112 enriched inoculated broths from routine screenings.

Statistical methods:

Sensitivity, specificity, PPV and negative predictive values (NPV) were calculated for the results of the Xpert assay performed directly on Eswab medium as well as on enriched inoculated broths. The 95% confidence intervals were calculated using the Wilson 95% confidence interval including continuity correction [20].

RESULTS:

Out of 235 Eswabs, 157 were negative in the Xpert *vanA/vanB* assay and confirmed to be true-negative for VRE according to our definitions (Table 1). In these 235 Eswabs no *vanA* VRE was found. A total of 78 Eswabs were *vanB* VRE positive according to the assay, of which 32 were confirmed to be true-positive for *vanB* VRE according to our definition. Moreover, all 32 isolates were identified as *E. faecium* and had a typical VanB phenotype by VITEK®2 susceptibility testing. The MIC values is these 32 isolates ranged from 8mg/L to >32mg/L

for vancomycin, for teicoplanin all MIC values were <0.5 mg/L. The other 46 Eswabs were positive according to the assay, but no VRE could be confirmed using our gold standard and these were considered to be false-positive. Therefore, the Xpert assay on Eswabs resulted in a sensitivity, specificity, PPV and NPV of 100%, 77.3%, 41% and 100%, respectively (Table 2).

Table 1: Xpert *vanA/vanB* assay results using Eswabs and inoculated enriched broths with CT cut-off values for PCR positivity of ≤ 36 and ≤25 respectively, in relation to true VRE positivity and negativity.

	Eswabs (CT cut-off value ≤ 36)			**Inoculated enriched broths (CT-cut-off value ≤25)**		
	VRE positive*	**VRE negative***		**VRE positive***	**VRE negative***	
Xpert assay positive	32	46	78	31	0	31
Xpert assay negative	0	157	157	1	203	204
Total	32	203	235	32	203	235

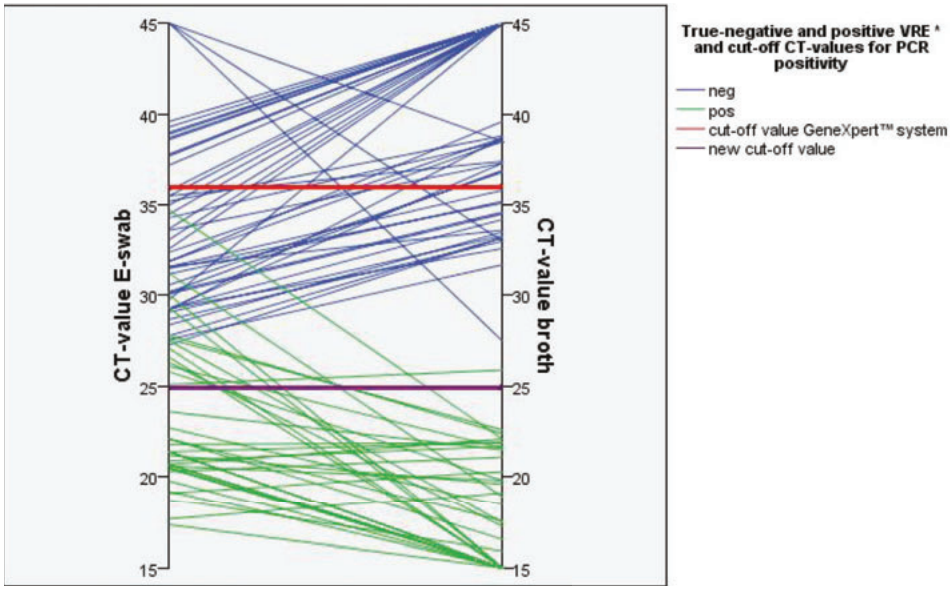
* See Material & Methods and Figure 1 for definitions.

Table 2: Sensitivity, specificity, PPVs and NPVs (95% confidence interval) of Eswab and inoculated enriched broth used in the Cepheid Xpert *vanA/vanB* PCR.

Method	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**
Eswab (CT cut-off value ≤ 36)	100 (86.7-100)	77.3 (70.8-82.8)	41 (30.2-52.8)	100 (97.2-100)
Inoculated enriched broth (CT-cut-off value ≤25)	96.9 (82-99.8)	100 (97.7-100)	100 (86.3-100)	99.5 (96.9-100)

Using the Xpert assay on enriched broths resulted in a decrease of CT-values for the majority (80.6%) of true-positive cases compared to their CT-values obtained directly from Eswabs. For true-negative cases the opposite was observed for 94.7% of the samples (Figure 2). Because of the observed decline in CT-values of the broths we adjusted the cut-off value for PCR positivity of the Xpert assay on broth to ≤25. Sensitivity, specificity, PPV and NPV were recalculated and were 96.9%, 100%, 100% and 99.5%, respectively (Table 1 and 2). By using a cut-off value of ≤25 no false positive results were found, however, one true-positive VRE was missed (CT-value 25.9). Therefore, we defined a "gray-zone" for samples with a CT-value between 25-30 that require confirmation by culture. In this case, patients were not cohorted with VRE-positive persons until these samples were confirmed by culture. CT-values of >30 were considered to be true-negative. Importantly, our algorithm was prospectively tested using 112 enriched inoculated broths. We found 80 true-negative samples, 31 true-positive samples and one sample with a CT-value of 28.4 which required confirmation. The new algorithm resulted in a sensitivity, specificity, PPV and NPV of 100% for all.

Figure 2: Dynamics between CT values acquired by performing the Xpert vanA/vanB assay directly on ESwabs (left) and on enriched inoculated broths (right). One line represents one sample. Blue lines represent samples with confirmed true-negative VRE cultures. Green lines represent samples with confirmed true-positive VRE cultures. The red line indicates the cutoff CT value for PCR positivity used by the Cepheid GeneXpert system (≤36). The purple line indicates the new cutoff CT value for PCR positivity (≤25). In this figure CT values of 45 indicate that no amplification was detected (negative test).



* See Material & Methods and Figure 1 for definitions.

DISCUSSION:

In this study we evaluated the use of the Xpert *vanA/vanB* assay on rectal swab specimens and on enriched inoculated broths for the detection of *vanB* VRE. By using enriched broths combined with a new cut-off CT-value of ≤ 25 for PCR positivity, the PPV for VRE detection increased from 41% to 100%. As shown in this study CT-values ≤ 25 acquired from enriched broth can be considered as true-positive. For broths with CT-values between 25-30, we recommend to confirm this by culture. CT-values of >30 appeared to be true-negative.

VRE detection remains difficult and open for discussion regarding the best method to be used for the most reliable results. We are aware of the fact that use of feces is superior to a rectal swab as used in this study. However, use of feces is less practical for the clinicians during an outbreak screening. Some studies consider PCR-positive specimens to be true-positive even when results could not be confirmed by culture [21]. Nevertheless, we choose culture based methods after broth enrichments for our negative and positive VRE definitions and consider these methods to be the most valid in this study design.

The use of enriched broths containing amoxicillin still enables ARE to grow, whereas the growth of amoxicillin sensitive bacteria including anaerobes, like *Clostridium species,* are inhibited. These are the most important gut microorganisms that would otherwise interfere with the assay as they may contain non-enterococcal *vanB* genes [8-10]. A limitation of using amoxicillin is that the growth of *E. faecalis* is also inhibited. Therefore, it would be worthwhile to test the use of another agent such as metronidazole instead of amoxicillin in enriched broths. However, as noted earlier, the majority of VRE outbreaks are typically caused by ARE that acquired resistance for vancomycin [15, 16].

All *vanB* VRE isolates found in this study had a typical VanB phenotype as determined by VITEK®2. Remarkable, in this study no *vanB* VRE expressing low vancomycin MIC values were detected, although these strains have been found in our hospital in the past years. Since no vancomycin was added to the broth, we are convinced that detection of these *vanB* VRE strains were adequately performed. In addition, we observed that these strains grow on VRE Brilliance agars as was also shown by others [12].

As a consequence of using enriched broths instead of direct rectal specimens, results will become available 24 hours later. On the other hand, by using this method the PPV increases from 41% to 100% which is essential for right decision making with respect to infection prevention. To control an outbreak it is crucial to cohort true-negative patients apart from true-positives. An ongoing outbreak might require closure of the ward which has a high financial impact and subsequently an enormous impact on patient care. Samples with CT-values between 25-30 will take another 24-48 hours, though only the minority of samples in our study were amongst these values (0.6% of all samples). Therefore, the use of the Cepheid's Xpert *vanA/vanB* assay on inoculated enriched broths with an adjusted CT-value for PCR positivity can be considered as an useful and rapid tool for the detection of *vanB* VRE.

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Transparency declarations

None to declare

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